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Asymmetric Hybrid Formation Revealed by Artificial Crossing Experiments between *Asplenium setoi* from the Ogasawara and Ryukyu islands, Japan

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The extent of reproductive isolation in various organisms increases with genetic distance. It was suggested that *Asplenium setoi* in the Ogasawara and Ryukyu islands were genetically differentiated and it was suspected that reproductive isolation may exist between them. In this study, *rbcL* sequences of the plants from these two localities were determined. They were found to have a 5-bp difference out of 1,203 bp. Artificial crossing experiments were also performed between *A. setoi* from the two localities to elucidate the extent of reproductive isolation between them. Although hybrids were obtained, the rate of hybrid formation differed between the reciprocal crosses. Twenty-three hybrids from 80 crosses were obtained when the maternal plants were from Ogasawara and the paternal plants were from Ryukyu. In the opposite combination of 80 crosses, only two hybrids were obtained. Thus, hybrids were easily formed only when plants from Ogasawara were the maternal parent. *Asplenium setoi* from the Ogasawara and Ryukyu islands may be bound for speciation.

Key words: artificial crossing experiments, Aspleniaceae, *Asplenium setoi*, Ogasawara, *PgiC*, *rbcL*, reproductive isolation, speciation

Although the species is the most basic taxonomy unit, it is a difficult term to define. The biological species concept (Mayr *et al.* 1942) is the most commonly accepted definition of species. It defines species as a group of populations that actually or potentially interbreed in nature and are reproductively isolated from other such groups. Following this definition, reproductive isolation is essential to consider the process of speciation and the origin of species. Even when attempting to recognize species, determining the extent of reproductive isolation is important. Many taxa reveal that reproductive isolation increases with genetic distance, which can be expected to increase with time because of divergence or speciation. Coyne & Orr (1989, 1997) analyzed 171 pairs of species of *Drosophila* for which information regarding the extent of reproductive isolation and allozyme-based estimates of genetic divergence, calculated as Nei's genetic distances, were available. The results indicated that the extent of both pre- and post-zygotic isolation linearly increased with genetic distance. Mendelson (2003) measured the extent of pre-mating isolation between seven species pairs of darter fish (*Etheostoma*), and those of post-mating isolation in nine of these species pairs. The extent of pre-mating isolation was found to be proportional to genetic distance that was calculated from nucleotide sequences of cytochrome *b*. Scopece *et al.* (2007) performed interspecific hand pollination experiments among five genera of Mediterranean or-

chids, some of which attract pollinators by mimicking nectariferous flowers and others by mimicking female insects. They reported that the extent of post-mating, post-zygotic isolation among food-deceptive species was positively related to the genetic distance among species, which was calculated on the basis of ribosomal ITS sequences. Thus, it has been demonstrated that the extent of reproductive isolation and genetic distance is often correlated.

In taxonomy, although each species is usually recognized from other species by differences in gross morphology, speciation is not always accompanied by morphological changes. In animals that visually attract mates and in flowering plants that attract pollinators with their flowers, different biological species tend to be easily recognized by their morphological differences. Their different morphologies are directly related to their reproductive isolation. In contrast, different biological species, which have neither eyes nor require the help by animals with eyes, are not necessarily morphologically different because such differences do not confer adaptive merits (Paris 1989). Therefore, in organisms such as ferns (Paris 1989, Yatabe *et al*. 2001, Masuyama *et al*. 2002) or fungi (Gac *et al*. 2007, Sato *et al*. 2007, Sato & Murakami 2008), it is often difficult to distinguish between closely related species based only on differences in appearance. Closely related species that are reproductively isolated but morphologically indistinct are referred to as cryptic species. In other words, pteridophytes and fungi are expected to have many cryptic species.

Asplenium sect. *Thamnopteris* C.Presl (Aspleniaceae) comprises a group of species of epiphytic ferns. Holttum (1974) defined the section as including species with sessile, simple fronds attached to the caudex in a very close spiraland with submarginal veins connecting the lateral veins. He recognized 15 species in sect. *Thamnopteris*. Among them, *A. nidus*, the type species of the section, is the most widely distributed in eastern Africa, Madagascar, the Mascarene islands, Sri Lanka, India, Indochina, southwestern China, the Malesian region, Solomon islands, Tahiti, and Hawaii. Because of its wide range of distribution and very simple frond morphology, *A. nidus* was suspected of containing many cryptic species.

Murakami *et al*. (2000) determined the sequences of the maternally inherited *rbcL* gene encoded in the chloroplast DNA and found three different *rbcL* types in plant samples of *Asplenium. nidus* from Mt. Halimun National Park. Yatabe *et al*. (2001) performed reciprocal artificial crossing experiments among the three *rbcL* types and found them to be reproductively isolated, thus, clearly revealing that at least three cryptic species are contained in *A. nidus*.

Yatabe *et al*. (2009) conducted artificial crossing experiments using 16 pairs of *rbcL* types of *Asplenium nidus* and possibly related species that were widely collected in Southeast Asia to clarify the relationship between the extent of reproductive isolation and genetic distances in the *A. nidus* complex. They calculated the genetic distance of each pair on the basis of their *rbcL* sequences using Kimura's two parameter method (Kimura 1980), then assessed the correlation between the extents of reproductive isolation and genetic distance. As a result, the number of hybrid offsprings obtained between the fern samples negatively correlated with genetic distances. No reproductive isolation was observed between individuals of the same *rbcL* type from distant localities, including Bogor, Indonesia and Iriomote Island, Japan, which are at least 4,000 km apart. They concluded that the extent of reproductive isolation was proportional to genetic distance in the *A. nidus* complex.

Yatabe *et al*. (2009) also reported that hybrids were obtained only in one of the two reciprocal crosses in the *Asplenium nidus* complex in which the genetic distances were 0.006 and 0.012. Hybrids were not produced when genetic distances were >0.013. Thus, asymmetric reproductive isolation tends to be occur when genetic distances are intermediate between no reproductive isolation and complete reproductive isolation.

Asymmetric reproductive isolation has been observed in other organisms as well. Tiffin *et al*. (2001) analyzed data from interspecific hand pol-

lination experiments among 14 genera of angiosperms and showed that asymmetric reproductive isolation is common in various taxa of angiosperms. Van der Velde & Bijlsma (2004) reported highly asymmetric reproductive isolation between the closely related bryophytes, *Polytrichum commune* and *P. uliginosum*. They analyzed diploid sporophytes from a sympatric population of two species using microsatellite markers and demonstrated that hybrid sporophytes were formed only when *P. uliginosum* was the maternal parent. Asymmetric reproductive isolation also occurs in invertebrates (Kaneshiro 1980, Wiwegweaw *et al*. 2009, Bath *et al*. 2012), vertebrates (Arnold *et al*. 1996, Bolnick & Near 2005, Whiteman *et al*. 2005, Devitt *et al*. 2011, Polačik *et al*. 2011) and ascomycetes (Dettman *et al*. 2003).

The asymmetric formation of hybrids has been reported also in several groups of ferns other than *Asplenium* sect. *Thamnopteris*. Vogel *et al*. (1998) identified the maternal parent of 97 individuals of *A.* ×*alternifolium*, which is a natural hybrid between *A. septentrionale* and *A. trichomanes*, on the basis of the genotypes of their chloroplast *trnL-F* regions. They found that *A. septentrionale* was the female parent of 94 hybrid individuals. *Asplenium trichomanes*, an inbred species, is unlikely to accept sperms from other individuals, whereas the outcrossing *A. septentrionale* is usually fertilized by non-self sperm. The authors presumed that such a difference in the breeding system may have caused the asymmetry. Testo *et al*. (2015) collected *Dryopteris* ×*triploidea*, a hybrid between *D. carthusiana* and *D. intermedia,* and *D.* ×*boottii*, a hybrid between *D. intermedia* and *D. cristata*, and determined the sequences of their chloroplast *trnL-F* regions. Consequently, 64 of 73 individuals of *D.* ×*triploidea* contained chloroplast DNA of *D. carthusiana* and 54 of 70 individuals of *D.* ×*boottii* contained chloroplast DNA of *D. cristata*. They also assessed sperm motility of the three parental species and reported that sperm of *D. intermedia* swam faster and lived longer than sperm of the other parental species. Furthermore, they evaluated antheridiogen production and the response to it in the three species and found that it induced the formation of antheridia in conspecific young gametophytes of *D. intermedia*, but not in the other two species. They concluded that such a response might enable *D. intermedia* to predominantly serve as the paternal parent in hybrids. Asymmetric hybridization is commonly observed in ferns and several hypotheses have been proposed to explain such phenomena.

Three species are recognized in *Asplenium* sect. *Thamnopteris* in Japan: *A. antiquum* Makino, *A. nidus* L. and *A. setoi* N.Murak. et Serizawa. All three were reported to be tetraploids (*A. antiquum*, Kawakami 1970, Dong 2011; *A. nidus*, Kawakami 1970, Yatabe *et al*. 2001, Dong 2011; *A. setoi* = *A. australasicum* in Iriomote Island, Japan, Nakato 1987). Among them, *A. setoi*, a species with a keeled costa, was recognized as *A. australasicum* (J.Sm.) Hook. (Iwatsuki 1995), a species closely related to *A. nidus.* Murakami *et al*. (1999) examined the intraspecific variation in allozymes and *rbcL* sequences in Japanese plants

TABLE 1. Voucher information for material of *Asplenium setoi* used in this study.

ID	Locality	Collector	Specimen No. (MAK)	Acc. No. (*rbcL*)	Acc. No. (*PgiC*)
HAHA-001	Mt. Chibusa, Haha Island, Ogasawara islands,TokyoPref., Japan	*K. Yamada*	*427902*	LC137791	
HAHA-002†	Mt. Chibusa, Haha Island, Ogasawara islands, Tokyo Pref., Japan	*K. Yamada*	*431582*	LC137789	LC137907 LC137908
HAHA-075†	Mt. Sekimon, Haha Island, Ogasawara island, Tokyo Pref., Japan	*S. Kato*	*385336*	LC137790	LC137907
IRI-187	Urauchi River, Iriomote Island, Ryukyu islands, Okinawa Pref., Japan	*K. Yamada*	*391126*	LC137786	
IRI-263†	Urauchi River, Iriomote Island, Ryukyu islands, Okinawa Pref., Japan	*K. Yamada*	*405366*	LC137787	LC137905 LC137906
IRI-270†	Urauchi River, Iriomote Island, Ryukyu islands, Okinawa Pref., Japan	*K. Yamada*	*405367*	LC137788	LC137905

† Individuals used for artificial crossing test.

of sect. *Thamnopteris* and compared them with plants from other paleotropical localities to determine the natural units of species. They discovered that the Japanese *Asplenium. setoi* [as *A. australasicum*] was genetically distinct from *A. australasicum sensu* Holttum (1974), which was originally described from an individual from Australia, and is distributed in both Australia and the South Pacific area. They then described the plants in Japan that have been called *A. australasicum* as a new species, *A. setoi*, the type locality of whici is Iriomote Island, Okinawa Prefecture, Japan.

Moreover, Murakami *et al*. (1999) reported that *Asplenium setoi* from Ogasawara and Ryukyu Islands have different *rbcL* sequences. They determined 1194 bp of *rbcL* sequences and identified seven different sites between the plants from the two localities. As previously described, species of sect. *Thamnopteris* whose genetic distances are >0.004 or that have >5 different sites in 1191 bp of *rbcL* sequences can be reproductively isolated (Yatabe *et al*. 2009). Therefore, *A. setoi* from the Ogasawara and Ryukyu islands can be reproductively isolated, and may represent distinct biological species. Sequencing techniques in the 1990s were not sufficiently accurate, however, to calculate the exact genetic distance between species on the basis of DNA sequences. It is therefore necessary to re-examine the differences in their *rbcL* sequences.

In contrast to Ryukyu islands, which are continental islands that have occasionally been connected to the Asian mainland, the Ogasawara islands are oceanic islands that are located 1,000 km south of the mainland of Japan. Despite their relatively small area and limited environmental diversity, they have a unique endemic flora. The percentage of endemism is approximately 45% for vascular plants and 23% for pteridophytes (Toyoda 2014). Their ancestors are considered to have arrived from the Ryukyu islands, Southeast Asia, Polynesia, and Micronesia through long distance dispersal events (Toyoda 2014). Several endemic species are adapted to the special dry environments in the Ogasawara islands (Ito 1998). It is no wonder that an unknown cryptic species of sect. *Thamnopteris* migrated from a distant locality and has evolved into a distinct biological species within the archipelago.

In this study, we performed artificial crossing experiments between *Asplenium setoi* from the Ogasawara and Ryukyu islands and quantitatively examined the extent of reproductive isolation between them. This study aimed to determine whether populations of *A. setoi* from the Ogasawara and Ryukyu islands are reproductively isolated.

Materials and Methods

Plant materials

Fresh green leaves of *Asplenium setoi* with mature sori were collected from Ryukyu islands, Okinawa Prefecture and Ogasawara islands, Tokyo Prefecture, Japan, from 2008 to 2014. The collected localities and other voucher information are shown in Table 1. At least two mature leaves were collected from each individual, one of which was used for DNA extraction and spore collection. Spores were collected in clean paper envelopes from air-dried leaves with sori. A piece of the leaf (approximately 3×3 cm) per sample was dried in silica gel for DNA extraction. Another leaf was dried and kept in the Makino Herbarium (MAK) in Tokyo Metropolitan University as a voucher specimen.

Three individuals of *Asplenium setoi* from Ogasawara (HAHA-001, HAHA-002 and HAHA-075) and from Ryukyu (IRI-187, IRI-263 and IRI-270) were used for sequencing of *rbcL* gene. For sequencing of *PgiC* gene, only parental individuals for artificial crossing experiments (HAHA-002 and HAHA-075 from Ogasawara, and IRI-263 and IRI-270 from Ryukyu) were used for analysis. For *rbcL* sequencing, total DNA was extracted using 2×hexadecyl trimethyl ammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 1.4M NaCl, and 20 mM EDTA) according to the method of Doyle & Doyle (1987). For *PgiC* sequencing, total DNA was extracted using DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) because DNA of higher purity is needed.

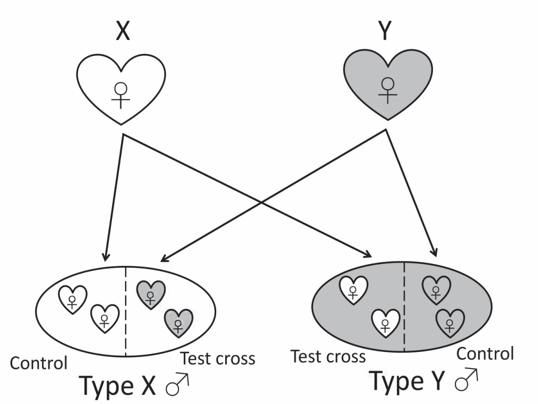


FIG. 1. Method of crossing experiments between Types X and Y of *Asplenium setoi* in this study. Detailed explanation is provided in Materials and Methods.

PCR amplification and sequencing of rbcL *gene*

The *rbcL* fragments were amplified by polymerase chain reaction (PCR) using the primers aF and cR that were developed by Hasebe *et al*. (1994). BioTaq (Bioline, London, UK) was used to amplify the DNA fragments using a GeneAmp PCR System 9700 (Thermo Fisher Scientific, Waltham, USA). The 20 μl reaction mixture comprised 10 μl of 2×Ampdirect Plus (Shimadzu, Kyoto, Japan), 0.5 U BioTaq, 0.2 μl of 50 μM forward primer, 0.2 μl of 50 μM reverse primer, 9 μl of sterilized distilled water, and 0.5 μl of template DNA eluate. Thermocycling programs entailed an initial denaturation step (95°C for 10 min) followed by 45 denaturation, annealing, and elongation cycles (94°C for 30 s, 53°C for 30 s and 72°C for 3 min, respectively) and a final elongation step (72°C for 7 min). The amplified fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN) and were used as templates for direct sequencing.

Sequencing reaction mixtures were prepared using a BigDye terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, USA). The primers aF, aR, cF and cR (Hasebe *et al*. 1994) and OT-NP1 and OT-2PR (Murakami *et al*. 1999) were used for cycle sequencing. The reaction mixtures were analyzed using an ABI PRISM 3100 or 3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA). The obtained sequences were assembled using ChromasPro version 1.34 (Technelysium, South Brisbane, Australia) and aligned using MEGA6.06 (Tamura *et al*. 2013). Genetic distances were calculated using Kimura's two parameter model.

Development of nuclear PgiC *marker*

New primers for the PCR amplification of the nuclear gene coding the cytosolic phosphoglucose isomerase (*PgiC* gene) of Aspleniaceae named Asp14F (5′-CTTCTGAGTGTTTGGAATGT-3′) and Asp16R (5′-TCCCCAAAATCAATTTCACC-3′), which are homologous to 14F and 16R that were prepared by Ishikawa *et al*. (2002) for *Dryopteris* (Dryopteridaceae), were developed on the basis of the transcriptome data of *Asplenium nidus* and *A. platyneuron* that were obtained from the 1KP Project (Matasci *et al*. 2014). Additional new primers Asp15F (5′-CTTGCTCCACATATACAACA-3′) and A481R (5′-GGTGTTCCTCCTTCATACGA-3′) were also developed in this study. Asp15F was developed based on the nucleotide sequence of the homologous sites of 15F (Ishikawa *et al*. 2002) in *A. trichomanes* that was reported by James *et al*. (2008).

PgiC fragments of *Asplenium setoi* were amplified using the Asp15F and A481R primers. The reaction mixture comprised 0.6 μl forward primer, 0.6 μl reverse primer, 5 μl PrimeSTAR Max Premix (Takara, Kusatsu, Japan), 3.3 μl SDW, and 0.5 μl template DNA that was extracted from the leaf samples. Touchdown PCR was conducted on a GeneAmp PCR System 9700 according to the following cycling program: 94°C for 5 min, 94°C for 1 s, 65°C for 5 s and 72°C for 20 s, followed by 10 cycles at decreasing annealing temperatures in decrements of 1°C per cycle, then 45 cycles of 1 s at 94°C, 5 s at 55°C, 10 s at 72°C and a final extension at 72°C for 7 min.

Because nuclear genes are inherited from both parents, and it is difficult to read the sequences of heterozygous individuals, PCR single strand conformation polymorphism (SSCP) analysis was conducted to separate allelic sequences according to the method of Hori *et al*. (2014). The PCR products were purified using Illustra ExoSTAR 1-Step (GE Healthcare, Little Chalfont, UK) and were used as templates for direct se-

TABLE 2. Differentiated sites in nucleotide sequences of *rbcL* (a) and *PgiC* (b) in *Asplenium setoi*. Positions of polymorphic nucleotide sites are indicated in bp from starting point of complete gene in *rbcL* and of obtained sequences in *PgiC*.

(a) *rbcL*

Position	68	279	336	402	810
IRI 187	C	G	C	G	G
HAHA 001	A	A	T	A	A

(b) *PgiC*

Position	57	69	70	77	78-81	100
IRI-263-1, IRI-270	T	C	A	C	-	T
IRI-263-2	T	C	A	G	-	T
HAHA-002-1,HAHA-075	-	T	C	C	GTAT	G
HAHA-002-2	-	T	C	C	GTAT	G

Position	129	230	245	278	311	399
IRI-263-1, IRI-270	G	A	A	C	C	A
IRI-263-2	G	A	A	C	C	A
HAHA-002-1, HAHA-075	A	T	G	T	T	T
HAHA-002-2	A	T	G	T	T	A

Position	413	416	430	452	536	571
IRI-263-1, IRI-270	A	C	C	T	G	T
IRI-263-2	A	C	C	T	G	T
HAHA-002-1, HAHA-075	C	C	T	T	A	G
HAHA-002-2	C	-	T	C	A	G

Position	575	633
IRI-263-1, IRI-270	A	C
IRI-263-2	A	C
HAHA-002-1, HAHA-075	G	T
HAHA-002-2	G	T

quencing. The primers, Asp15F and A481R were used for cycle sequencing. Thermocycling was conducted using a GeneAmp PCR System 9700 according to the same program followed for sequencing of *rbcL*.

For genotyping the offspring obtained in artificial crossing experiments (mentioned later), total DNA was extracted from silica gel-dried samples of juvenile sporophytes using the DNeasy Plant Mini Kit and amplified by touchdown PCR using the primers Asp14F and Asp16R. PrimeSTAR was used for amplifying the gene. The primers, Asp14F, Asp15F, and Asp16R were used for cycle sequencing. The cycling program of touchdown PCR and cycle sequencing was the same as that previously mentioned. The reaction mixtures were analyzed using an ABI PRISM 3130 Genetic Analyzer. The obtained chromatogram files were analyzed by ChromasPro version 1.34. Individuals with double peaks on the sites of the polymorphisms were determined as hybrids.

Artificial crossing experiments

Spores of the parental individuals of *Asplenium setoi* (IRI-263, IRI-270, HAHA-002 and HAHA-075) were sown on agar-solidified nutrient medium containing Parker's macronutrients and Thompson's micronutrients (Klekowski 1969) in plastic plates 9-cm in diameter (AGC Techno glass, Yoshida, Japan). After cultivation at 25°C for 6 weeks, female gametophytes with archegonia were usually obtained, although gametophytes with antheridia were seldom found. Forty female gametophytes were transplanted to the same nutrient medium in the 9-cm plates. Additional spores from the same individuals of the sporophytes were sown around the remaining gametophytes in the plates after removing the forty

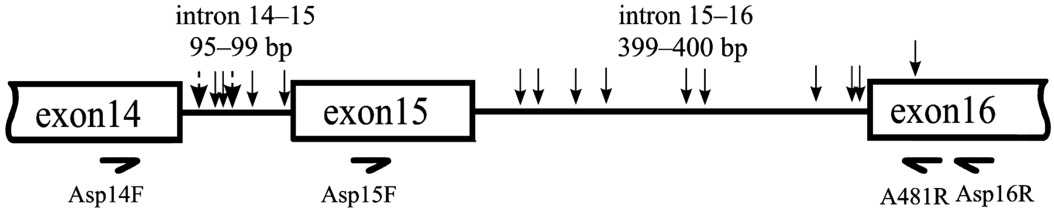


FIG. 2. Positions of newly developed primers and useful nucleotide sites to distinguish *Asplenium setoi* from Ogasawara and Ryukyu islands. Relative position of three exons and two introns and those of four primers used in this study are shown. Polymorphic sites are shown as solid and dashed arrows because of substitutions and indels, respectively.

gametophytes. Gametophytes formed from resown spores develop as males because of antheridiogens secreted by the mature female gametophyte (Näf *et al.* 1975, Haufler & Gastony 1978). They were cultivated at 25°C for another 1 month.

The artificial crossing experiments were performed following the method of Yatabe *et al.* (2009). The artificial crosses between hypothetical Types X and Y are illustrated in Fig. 1. Twenty female gametophytes with archegonia of Type X were transferred to the dish that was flooded with distilled water, in which many sperm swam from the gametophytes with antheridia of Type Y. The same number (20) of gametophytes of Type X were also transferred to a dish of the Type X sperm as a control. These procedures were reciprocally conducted and the dishes were left overnight to insure fertilization. The 80 treated gametophytes in one set of the crossing experiments were then placed in new 9-cm plastic plates with the same agar medium. Two to three months after artificial crossing, gametophytes bearing juvenile sporophytes were counted. If no sporophytes were obtained in either of the controls, the dataset was discarded. Leaves of all offspring sporophytes obtained from the crossing experiments, including the controls, were preserved in silica gel for further DNA analysis to determine their genotypes because sporophytes produced from the crossing treatments may have been generated through intragametophyte selfing or outcrossing between floating female gametophytes with antheridia.

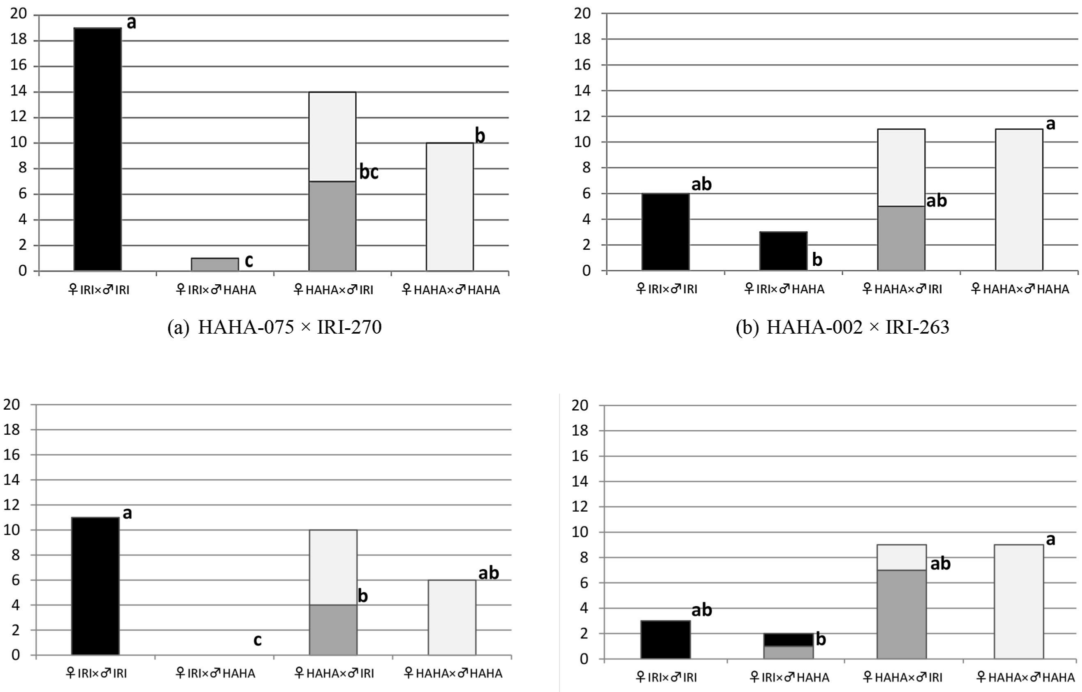
We used Tukey-Kramer's multiple comparison test to compare the number of gametophytes bearing sporophytes in each test cross and controls using R v. 3.2.2.

Results

Sequencing of chloroplast rbcL *gene and nuclear* PgiC *gene*

The 1203-bp nucleotide sequences of the *rbcL* gene were determined for *Asplenium setoi* from Ogasawaraand *A. setoi* from Ryukyu. The nucleotide sites that differed between the individuals from Ogasawara and those from Ryukyu are shown in Table 2(a). The individuals of *A. setoi* from Ogasawara had the same *rbcL* sequence. With respect to *A. setoi* from Hahajima and Ryukyu, a 5-bp difference was observed in the analyzed region of the *rbcL* gene. Although the amount of genetic difference between them was less than that reported in a previous study (Murakami *et al.* 1999), *A. setoi* from the two localities clearly had different *rbcL* sequences. The genetic distance based on their *rbcL* sequences using Kimura's two parameter method was 0.004.

The 666-bp sequences of the nuclear *PgiC* gene were also determined in this study. Two types of sequences of the *PgiC* gene were detected in individuals of *Asplenium setoi* from Ryukyu and an additional two types in *A. seto*i from Ogasawara. The 16 nucleotide sites of *PgiC* were different between individuals from Ogasawara and Ryukyu. The nucleotide sites that were useful for distinguishing individuals from Ogasawara and Ryukyu are shown in Table 2(b) and Fig. 2. *Asplenium setoi* is a tetraploid and is expected to



(a) HAHA-075 × IRI-270

(b) HAHA-002 × IRI-263

(c) HAHA-002 × IRI-263

(d) HAHA-002 × IRI-263

FIG. 3. Number of juvenile sporophytes obtained in each crossing experiment between individuals of *Asplenium setoi* from Ogasawara and Ryukyu islands. Labels indicate pairs of parental sporophytes used in each crossing experiment. Black bars = homozygous plants of which *PgiC* type is Ryukyu; pale gray bars = homozygous plants of which *PgiC* type is Ogasawara; thick gray bars = hybrids. Letters indicate statistically significant difference in p value <0.05.

have two homoeologous *PgiC* loci. It is because the ancestor of *Asplenium* sect. *Thamnopteris* was suggested to be tetraploid (Perrie & Brownsey 2005). The two loci must have diverged after polyploidization. If both of these two *PgiC* loci were amplified, fixed heterozygosity should be observed. However, we obtained homozygous individuals with either the Ogasawara or the Ryukyu types of *PgiC* in addition to the heterozygous ones. Therefore, our primers might have amplified only one of the duplicated loci.

Artificial crossing experiments

In total, 15 datasets were generated. Among them, four datasets with successful controls were obtained. One was a cross between IRI-270 and HAHA-075, and the others were crosses between IRI-263 and HAHA-002. The number of female gametophytes bearing juvenile sporophytes and those bearing hybrids between the two parents are given in Fig. 3. Eight hybrids were obtained from the HAHA-075 and IRI-270, parents for dataset (a). Among them, HAHA-075 was the maternal parent of seven hybrid sporophytes, and IRI-270 was the parent of one hybrid sporophyte.

The parental plants of other datasets were IRI-263 and HAHA-002. In datasets (b) and (c), hybrid sporophytes of which the maternal parent was IRI-263, were not found. In dataset (d), only one hybrid sporophyte, for which maternal parent was IRI-263, was found. In contrast, several hybrids of the maternal plant HAHA-002 were observed in every dataset. The number of sporophytes obtained in this study was 108. Twenty-three hybrids were obtained when the maternal plants were from Ogasawara. In the opposite combination of crosses, only two hybrids were

obtained. Statistical tests indicated that the rates of hybrid formation in reciprocal crosses were significantly different only in the dataset (c). However, the numbers of hybrids obtained tended to be lower when *A. setoi* from Ryukyu was the maternal parent. Moreover, when the maternal plants were from Ryukyu, the rate of hybrid formation tended to be lower than in the controls.

Discussion

Asymmetric hybrid formation observed in Asplenium setoi *and possible reproductive isolation*

In this study, *Asplenium setoi* from Ogasawara and Ryukyu was suggested to be at least partially reproductively isolated. Genetic differentiation was demonstrated not only in the chloroplast *rbcL* gene but also in the nuclear *PgiC* gene. Additionally, although hybrids between individuals of *A. setoi* from Ogasawara and Ryukyu were obtained in artificial crossing experiments, hybrid formation was asymmetric. Yatabe *et al.* (2009) reported that in two pairs of the *A. nidus* complex, in which genetic distances were 0.006 and 0.012, hybrid sporophytes were found in only one of the two crosses. They also reported that when the genetic distance of the parental plants was <0.001, the number of hybrids did not differ in the reciprocal combinations of maternal and paternal parents and freely crossed. Asymmetric hybrid formation in this study appears intermediate. Considering the genetic distance between the parents used in this study, asymmetric hybrid formation might be at an intermediate stage between free crossability and complete reproductive isolation.

The extent of reproductive isolation in species of *Drosophila* is correlated with the genetic distances calculated on the basis of allozyme markers. Asymmetric reproductive isolation is also frequently observed in *Drosophila*. Pairs with genetic distances ranging from 0.134 to 1.610 resulted in asymmetric crosses among all the pairs for which genetic distances ranged from 0.024 to 1.950 (Kaneshiro 1976, Lachaise *et al.* 1986, Coyne & Orr 1989, Orr & Coyne 1989, Fontdevila *et al.* 1990, Marin *et al.* 1993, Coyne & Orr 1997, Nickel & Civetta 2009). Turelli & Moyle (2007) predicted that the magnitude of asymmetry increases with genetic distance between parental taxa, and then falls when one of the reciprocal crosses produces complete isolation. They demonstrated that such reproductive isolation pattern occurs in centrarchid fishes (Centrarchidae) and three genera of angiosperms (*Glycine, Silene*, and *Streptanthus*). Asymmetric reproductive isolation observed in the *Asplenium nidus* complex also fits the pattern proposed in these previous studies.

Yatabe *et al.* (2009) later reported that abnormal spore formation and asymmetric hybrid formation were observed in the hybrid sporophytes between two parents of *Asplenium* sect. *Thamnopteris* with a genetic distance of >0.006 in Kimura's two parameter. The parents in their studies were demonstrated to belong to different biological species as evidenced by F_1 hybrid sterility. Similar sterile hybrid sporophytes and post-zygotic isolation between individuals of *A. setoi* from Ogasawara and Ryukyu was observed in this study. If post-zygotic isolation between populations of *A. setoi* from Ogasawara and Ryukyu is confirmed, the former should be treated as a species distinct from *A. setoi*, because the type locality is Iriomote Island, Ryukyu. Morphological differences between plants of *A. setoi* from Ogasawara and from Ryukyu have not been recognized. A new species might be described only on the basis of differences in *rbcL* sequence as is the case with *A. setoi* itself (Murakami *et al.* 1999).

As described in the introduction, asymmetric hybrid formation has been reported in various taxa of ferns, but in contrast to previous studies focusing on natural hybrids in their native habitat, a sufficient sperm supply was confirmed by successful fertilization in the control experiments in this study. Therefore, differences in breeding systems and the sex ratio of gametophytes were easily excluded as a cause of asymmetric hybridization. Consequently, asymmetric hybrid formation observed in *Asplenium setoi* from these two localities should be due to other factors including physical ones.

Turelli & Moyle (2007) explained that asymmetric postzygotic isolation often results from incompatibility between autosomes and uniparentally inherited genetic factors, including mitochondrial DNA, chloroplast DNA, maternal transcripts, and sex chromosomes. Among them, it is improbable that incompatibility among autosomes and sex chromosomes occurs in ferns, because ferns are hermaphroditic, even in the gametophytic generation, and do not have sex chromosomes. However, incompatibility due to other factors may be related to asymmetry in ferns.

Several possible explanations can be proposed for asymmetric hybrid formation observed in this study. Schneller (1979) reported that the archegonial mucilage of *Athyrium filix-femina* and *A. distentifolium* paralyze sperms of *Dryopteris filix-mas* before they penetrate the archegonial venter. Moreover, the archegonial mucilage of *D. filix-mas* exhibits a weak positive chemotactic influence on sperms of the two species of *Athyrium*. He assumed that differences in incompatibility in reciprocal crosses was due to differences in concentration of the chemotactic substances in the archegonial mucilage. Another explanation may be nuclear–cytoplasmic incompatibility. This is the widely accepted theory of asymmetric postzygotic isolation in angiosperms (Levin 1978, Tiffin 2001, Levin 2003). Schmitz & Michaelis (1988) proposed that nuclear–mitochondrial interaction causes asymmetric male sterility between *Epilobium hirsutum* and *E. montanum* (Schmitz 1988) after analyzing the transcription of mitochondrial genes in male sterile hybrids and their maternal parents. The proximate cause of such asymmetry in ferns may be revealed by observing fertilization or early developmental stages of zygotes.

Utility of nuclear DNA markers

Codominant nuclear markers are indispensable for performing artificial crossing experiments in ferns, because their gametophytes are hermaphroditic and may self-fertilize within a gametophyte. In this study, new primers compatible with the *PgiC* gene of *Asplenium* sect. *Thamnopteris* were developed and genotypes of juvenile sporophytes produced from artificial crossing experiments were determined using a sequence variation of the nuclear *PgiC* gene. In previous studies, allozyme markers have often been used for the parental determination of offspring in crossing experiments of ferns (Yatabe *et al*. 2001, Masuyama *et al*. 2002, Yatabe *et al*. 2009). Nuclear DNA markers have two advantages over allozyme markers. First, because DNA is much more stable than enzymes, the handling of plant samples is easier. Second, DNA data can be obtained even from a very small amount of samples, even from a single pollen-particle, by amplifying specific DNA fragments using PCR (Isagi & Suyama 2011). In future studies of *Asplenium nidus* complex, the nuclear *PgiC* marker developed in this study should be useful in revealing the developmental stages during which reproductive isolation occurs by detecting hybrid zygotes or small embryos.

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